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The etiologic agent of plague is the Gram negative bacterium *Yersinia pestis*. *Y. pestis* is a concern as one of the microorganisms with potential for use against civilian or military populations as a biological warfare/ biological terrorism agent. In that case, the pneumonic form of plague would be the most likely outcome. This form of plague is particularly devastating because of the rapidity of onset, the high mortality, and the rapid spread of the disease. Immunization against aerosolized plague presents a particular challenge for vaccine developers. The studies reported herein explore the ability of a novel adjuvant, designated LT(R192G), to promote the rapid development of long-lasting, high titer antibodies against a recombinant plague antigen (F1-V) and protection in a murine model. Subsequent studies will be performed in non-human primates. Different routes of administration are examined to test the hypothesis that heterologous boosting will be more effective than homologous boosting at increasing the magnitude and/or duration of the antibody response.

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INTRODUCTION

The etiologic agent of plague is the Gram-negative bacterium *Yersinia pestis*. *Y. pestis* is a concern as one of the microorganisms with potential for use against civilian or military populations as an agent of biological warfare or biological terrorism. In that case, the pneumonic form of plague would be the most likely outcome. This form of plague is particularly devastating because of the rapidity of onset, the high mortality, and the rapid spread of the disease. Immunization against aerosolized plague presents a particular challenge for vaccine developers. A number of potential subunit vaccine against plague have been evaluated for immunogenicity and protective efficacy. The two most promising are the *Y. pestis* proteins F1 and V. F1 is a capsular protein located on the surface of the bacterium and the V-antigen is a component of the Type III secretion system. In previous studies, combined immunization with native F1 and recombinant V (rV) in a two-dose regimen afforded full protection in mice against subcutaneous challenge with *Y. pestis* (24) and the anti-F1 and anti-V titers, especially of the IgG1 sub-class, correlated significantly with protection in BALB/c mice. Male and female CBA, C57/BL6 and CB6F1 mice were also protected against injected and aerosol challenge with *Y. pestis* following immunization with two doses of rF1 and rV (13). The combination or fusion of F1 and V has been has an additive protective effect in the murine model when compared to either antigen alone (4, 5, 12, 19, 21, 22). Heath et al. (12) reported construction of a an F1-V fusion consisting of the F1 protein fused at its carboxyl terminus to the amino terminus of the entire V-antigen. F1-V was shown to provide excellent protection against both subcutaneous and aerosol challenge and has the potential to provide protective immunity against pneumonic as well as bubonic plague due to either wild type $F1^{+}$ *Y. pestis* or to naturally occurring $F1^{-}$ variants.

Soluble protein-based vaccines, such as F1-V, are generally administered subcutaneously or intramuscularly in the presence of an aluminum salt adjuvant. For most proteins, this is an effective means of inducing serum antibody against the antigen (i.e., tetanus and diphtheria toxoid). Recently, a great deal of attention has been directed towards needle-free immunization strategies as alternative methods for vaccine delivery. Both mucosal (intranasal, oral, rectal) and transcutaneous immunization in the presence of an appropriate adjuvant have been shown to induce humoral and cellular immune response in both the systemic and mucosal compartments. Alternating routes for delivery of the priming dose and booster dose in immunizations, so called "prime-boost" strategies have also been examined for the ability to induce high-titer, long-lasting humoral responses and have the potential direct or redirect the immune response to one compartment or another. This may be particularly useful for development of vaccines against agents that may be delivered by aerosol, where the respiratory mucosa would be the first point of productive contact between the organism and the host.

In the current contract, we examine different prime/boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the ability of recombinant F1-V to promote the development of long-lasting, high titer antibodies. We also examine the effect of different prime/boost regimes on the compartmentalization of the ensuing immune response. For parenteral immunization, F1-V is adsorbed to aluminum hydroxide, which is commonly used as an adjuvant for parenterally administered vaccines. Mucosally and transcutaneously administered vaccines are usually not immunogenic and also require the presence of an appropriate adjuvant. In the current studies, we utilize a mutant of the heat-labile enterotoxin of *Escherichia coli*,

designated LT(R192G), that has been shown to be effective when administered mucosally (orally, rectally, intranasally) or transcutaneously in a variety of animal models and in humans.

BODY

This project is organized into two Specific Aims that constitute the Technical Objectives of the proposal.

Specific Aim 1. Optimize the Immune Response to F1-V in a Murine Model. In the first specific aim, we examine the ability of LT(R192G) to function as an adjuvant for F1-V when delivered mucosally or transcutaneously and the ability of adjuvanted mucosal or transcutaneous immunization to serve as a booster for parenteral priming. The primary objective of this aim is to optimize immunization to achieve a rapid anti-F1-V antibody response of high titer and of long duration. Another objective of this aim is to determine if the antibody response to both antigens, F1 and V, is sustained. Aerosol challenge of immunized mice will be conducted to correlate the induction of serum and mucosal antibodies with protection. The optimum prime/boost regimen from these studies, as defined by antibody responses and confirmed by challenge, will subsequently be examined in Non-Human Primates (NHP).

Specific Aim 2. Evaluate the Immune Response to F1-V in Non-Human Primates. The second specific aim is to evaluate the optimum prime/boost regimen from Specific Aim 1 in nonhuman primates. The primary objective of this aim is to optimize immunization to achieve a rapid response of high titer and of long duration. Another objective of this aim is to determine if the antibody response to both antigens, F1 and V, is sustained. Animals will be followed monthly to determine their antibody response to parenteral F1-V and whether the optimum prime/boost regimen from Specific Aim 1 can enhance and extend that response. Since previous studies have suggested that F1-V administered parenterally is not optimally protective in NHP (Dr. Jeffrey Adamovicz, Personal Communication), immunized animals will be transferred to a USAMRIID designated facility for challenge.

During this reporting period, we successfully immunized all animals as specified in the approved Scope of Work for Specific Aim 1 and analyzed the serum and BAL responses through twelve-months post-primary immunization. Groups of 6-8 week old female Swiss Webster mice (Charles River Laboratories) were immunized twice (day 0 and day 28) with recombinant F1-V. Mice were immunized subcutaneously (SC), intranasally (IN), or transcutaneously (TCI) and then boosted by the same (homologous) or a different (heterologous) route as shown in the following table (Table 1).

Table 1: Immunization Schedule

Priming Dose	Boosting Dose at Day 28		
Subcutaneous (SC)	SC	IN	TC
Intranasal (IN)	SC	IN	TC
Transcutaneous (TC)	SC	IN	TC

Animal Immunizations and measurement of serum and bronchioalveolar lavage antibodies. Groups of 8-9 week old female Swiss Webster mice (Charles River Laboratories) were immunized once (day 0) or twice (day 0 and day 28) with the recombinant F1-V fusion

protein vaccine developed at the United States Army Medical Research Institute of Infectious Diseases (11). LT(R192G) was prepared in our laboratory by galactose-affinity chromatography as previously described (2). Mice were immunized SC, intranasally (IN), or transcutaneously (TC) and then boosted by the same (homologous) or a different (heterologous) route (Table 1). Mice immunized SC received 10 µg of recombinant F1-V adsorbed to 0.19 mg of an aluminum hydroxide adjuvant (2.0% Alhydrogel batch no. 3275; Superfos Biosector, Vedbaek, Denmark) in a final volume of 100 µl. Mice immunized IN received 5 µg of recombinant F1-V admixed with 5 µg LT(R192G) in a final volume of 9.6 µl in one nostril following brief exposure to Isofluorane. Mice previously anesthetized by intraperitoneal injection with Ketamine-Xylazine were immunized TC with 35 µg F1-V admixed with 25 µg LT(R192G) in a final volume of 50 µl applied to freshly shaved ventral skin. These doses were based upon preliminary studies in our laboratory and upon studies published elsewhere.

Mice were sacrificed by CO_2 inhalation in groups of 5 from each of the 3 primary immunization groups on day 28 and from each of the 9 homologous and heterologous prime - boost groups on days 59, 191, and 385. Blood was obtained from each animal by cardiac puncture. Lung lavage fluid was collected from each animal by exposing the trachea, making a small incision, inserting and securing an 18-gauge needle, and aspirating 1 ml of PBS three times before final withdrawal. Serum and bronchioalveolar lavage (BAL) fluid were examined for the presence of anti-F1-V, anti-F1 or anti-V antibodies by ELISA. Briefly, ELISA plates were coated with 0.1 µg per well of recombinant F1-V, F1 or V in 100 µl ammonium bicarbonate buffer. Following overnight incubation at 4C, plates were washed in PBS containing 0.05% Tween 20, and two-fold serial dilutions of the serum or BAL from immunized animals were applied. After incubation for 1 hour at room temperature, plates were washed and a 1:400 dilution of goat anti-mouse IgG or IgG1 labeled with alkaline-phosphatase was added and incubation continued for 1 hour at room temperature. Plates were washed and the substrate PNPP was added. The reaction was stopped with 2N NaOH and the plates were read at an optical density at 405 nm. For quantitative analysis, concentrations of serum and BAL anti-F1-V, -F1, or -V IgG or IgG1 were determined by nonlinear regression from a standard curve of mouse myeloma IgG1 (Sigma Chemical Co., Saint Louis, MO) serially diluted as a standard on each ELISA plate. The results obtained are expressed as the mean concentration ± SEM. Statistical analyses were performed using a one-way analysis of variance with the Bonferroni Multiple Comparison post-test.

Serum and BAL anti-F1-V IgG1 response following homologous prime - boost. The purpose of this group of experiments to was compare three different routes of immunization (IN, TC, SC) for the ability to induce high titer anti-F1-V serum and BAL antibody responses following one or two immunizations with the priming dose and booster dose delivered by the same (homologous) route. Mice were immunized once or twice with F1-V adsorbed to alum (SC) or admixed with LT(R192G) (IN, TC) and groups of five animals from each regime were sacrificed at days 28, 59, 191, and 385 post-primary immunization. Since previous studies by Williamson et al. (23) had shown that anti-F1 and anti-V IgG1 responses correlated significantly with protection, serum and BAL anti-F1-V IgG1 responses were determined by ELISA.

As shown in Figure 1 and Tables 2 and 3, a single primary immunization with F1-V by any of the three routes induced no (TC) or only a minimal (IN, SC) anti-F1-V serum or BAL antibody response at 28 days post-primary immunization. The maximum serum (207±82 µg/ml) and BAL

(531±163 ng/ml) anti-F1-V IgG1 response achieved following a single primary immunization was by SC delivery. There was no detectable serum or BAL anti-F1-V antibody response following a single TC immunization.

Table 2: Serum anti-F1-V IgG1 (μg/ml) ± SEM

	Day 28	Day 59	Day 191
IN	5±1		
TC	0		
SC	207±82		
IN x IN		519±114	417±103
IN x TC		461±116	363±131
IN x SC		1,124±268	1,454±358
TC x IN		97±55	41±6
TC x TC		53±12	55±41
TC x SC		412±124	473±187
SC x IN		702±93	700±226
SC x TC		868±228	1,678±298
SC x SC		779±132	859±91

Table 3: BAL anti-F1-V IgG1 (ng/ml) ± SEM

	Day 28	Day 59	Day 191
IN	23±23		
TC	0		
SC	531±163		
IN x IN		4,207±1,197	893±267
IN x TC		7,466±3,343	1,245±728
IN x SC		3,109±623	1,020±206
TC x IN		562±196	177±58
TC x TC		297±132	50±31
TC x SC		3,104±1687	471±172
SC x IN		1,973±399	2,211±1,038
SC x TC		9,336±3333	2,374±528
SC x SC		3,145±1050	1,244±190

Following a single homologous boosting dose administered at day 28, serum and BAL anti-F1-V IgG1 responses increased in all three groups with peak responses observed by day 59. Specifically, anti-F1-V responses after day 59 were never significantly ($p>.05$) greater than the day 59 responses within any group. The highest responses were observed following either IN or SC immunization, which were not different from one another at day 59 ($p>.05$). With respect to concentrations of anti-F1-V antibodies, both IN and SC regimes induced significantly higher levels of serum and BAL anti-F1-V IgG1 than did TC immunization with the same antigen ($p<.01$). To be clear, homologous TC prime - boost did induce significant levels of serum (53±12 μg/ml) and BAL (297±132 ng/ml) anti-F1-V IgG1 at day 59, just not to the same level as that obtained following either IN or SC homologous prime - boost.

With respect to duration of response, there was no significant decline in serum anti-F1-V IgG1 through 6 months post-primary immunization within groups of animals immunized either IN, TC or SC, while there was a significant decrease in BAL anti-F1-V IgG1 between two and six months. Specifically, the serum anti-F1-V response at day 191 was not significantly different from the serum anti-F1-V response at day 59 following either IN, TC or SC prime - boost

(p>.05). In contrast, the BAL anti-F1-V response declined between two and six months post-primary immunization. When comparing IN and SC homologous prime - boost, SC immunization induced higher and more sustained levels of serum, but not BAL, anti-F1-V than did IN prime - boost. Thus, the serum anti-F1-V IgG1 response at 191 days post primary immunization following SC prime boost (859±91 μg/ml) was significantly higher than that obtained following IN prime boost at day 191 (417±103 μg/ml) (p<.01).

With respect to distribution, both IN and SC prime - boost induce significant and sustained levels of both serum and BAL anti-F1-V IgG1.. Taken together, these results demonstrate that IN and SC immunizations are both effective and essentially equivalent for induction of serum and BAL anti-F1-V IgG1 responses when a single booster dose is administered by the same (homologous) route. TC homologous prime - boost was less effective than either IN or SC homologous prime - boost.

Serum and BAL anti-F1-V IgG1 response following heterologous prime - boost. Heterologous boosting offers the possibility of increasing the magnitude or duration of the immune response when compared to homologous boosting and may also influence the compartmentalization of that response. For these studies, animals received a primary immunization by one route and then a single booster dose by the same or an alternate route (Table 1). As above, groups of five animals from each regime were sacrificed at days 28 (no boost), 59, 191, and 385 post-primary immunization and examined for the presence of serum and BAL anti-F1-V IgG1 by ELISA.

As seen in Figure 2.A and Tables 2 and 3, animals primed IN and then boosted SC (circle) developed higher levels of serum anti-F1-V than did animals primed IN and boosted either IN (square) or TC (triangle) (p<.05). This IN-prime - SC-boost response remained elevated through six-months post-primary immunization and declined thereafter such that by day 385 there were no significant differences between groups. The serum anti-F1-V responses in animals primed IN and boosted IN (square) or TC (triangle) also remained elevated through six-months, although clearly at a lower level than that obtained with IN priming and SC boosting (Fig 2A). The BAL anti-F1-V IgG1 responses at day 59 for animals primed IN were highest in animals boosted TC (p<.05) when compared to animals boosted IN (Fig. 2.B) while IN and SC boosting of IN primed animals produced responses that were indistinguishable from one another. All BAL anti-F1-V IgG1 responses dropped to baseline by six months post-primary immunization.

Transcutaneous immunization is an attractive needle-free alternative to parenteral immunization and numerous studies have demonstrated that transcutaneous homologous prime - boost can be effective for induction of antigen-specific serum and BAL antibody responses (6-10). However, the effect of TC priming and heterologous boosting has not been widely studied. As shown in Figures 2C and 2D, animals primed TC and then boosted SC developed higher levels of serum (Fig. 2C) and BAL (Fig. 2D) anti-F1-V than did animals primed TC and boosted either IN or TC (p<.05). Animals primed TC and then boosted either TC or IN developed equivalent serum and BAL anti-F1-V responses.

Parenteral prime and boost is the more traditional route of vaccine delivery. In our studies, animals primed SC and boosted IN or TC had higher levels of serum anti-F1-V IgG1 through six-months post primary immunization than animals primed SC and boosted SC (p<.05) (Fig. 2E).

The highest level of BAL anti-F1-V following SC priming was obtained by TC boosting ($p<.05$) when compared to either IN or SC boosting (Fig. 2F). As with IN priming, the BAL responses were maximal at two-months post-primary immunization and declined significantly by six-months.

These findings demonstrate that the highest and most sustained levels of serum anti-F1-V IgG1were obtained following IN priming and SC boosting or SC priming and either IN or TC boosting, while the highest levels of BAL anti-F1-V IgG1were obtained following either IN or SC priming and TC boosting. These studies clearly demonstrate that heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1-V IgG1 responses. In no case was heterologous boosting inferior to homologous boosting.

F1- and V-specific IgG responses following heterologous prime - boost. In order to determine the effect of homologous and heterologous prime - boost on the serum response to both F1 and V, separate F1 and V ELISA assays were performed on sera from the two peak-time points for each group, days 59 and 191 post-primary immunization. For these studies, in contrast to the studies above where anti-F1-V IgG1 responses were determined, total IgG serum anti-F1 and anti-V responses were evaluated to obtain an assessment of the broader antibody response to each of the two antigens. As seen in Figure 3, anti-F1 and anti-V total IgG responses were highest in animals primed IN and boosted by any route when compared to animals primed TC or SC. This is an important observation since the correlate of protection is generally regarded to be serum anti-F1 or anti-V IgG1 and the higher magnitude of total IgG response following IN immunization may not be more protective. In general, serum anti-F1 and anti-V total IgG responses diminished over time, the notable exception being animals that were primed SC and boosted heterologously (IN or TC), which may have implications for long-term protection.

Protection against aerosol challenge. A number of studies have demonstrated a direct correlation between protection against challenge and the levels of serum anti-F1 or anti-V IgG1. Based upon the anti-F1-V IgG1 responses observed in homologous and heterologous prime – boost experiments shown in Figures 1 and 2 and Tables 2 and 3, 16 groups of ten Swiss Webster mice were immunized once or twice with F1-V alone or in combination with adjuvant by one of three routes (IN, TC, SC – see Table 4) at Tulane University and challenged by aerosol at USAMRIID with 70 LD50 of *Y. pestis* CO92 using a dynamic aerosol system within a Class III safety cabinet.

Table 4 – Aerosol Challenge Groups

Group	Prime Antigen	Prime Adjuvant	Boost Antigen	Boost Adjuvant
1- naive	-	-	-	-
2- IN x IN	5 μg F1-V	-	5 μg F1-V	-
3- IN x IN	-	5 μg mLT	-	5 μg mLT
4- IN x IN	5 μg F1-V	5 μg mLT	5 μg F1-V	5 μg mLT
5- IN x SC	5 μg F1-V	-	10 μg F1-V	-
6- IN x SC	-	5 μg mLT	-	10 μl Alhy
7- IN x SC	5 μg F1-V	5 μg mLT	10 μg F1-V	10 μl Alhy
8- SC x SC	10 μg F1-V	-	10 μg F1-V	-
9- SC x SC	10 μg F1-V	10 μl Alhy	10 μg F1-V	10 μl Alhy
10- SC x TCI	10 μg F1-V	-	35 μg F1-V	-

11- SC x TCI	10 μg F1-V	10 μl Alhy	35 μg F1-V	25 μg mLT
12- TCI x SC	35 μg F1-V	-	10 μg F1-V	-
13- TCI x SC	35 μg F1-V	25 μg mLT	10 μg F1-V	10 μl Alhy
14- IN	5 μg F1-V	5 μg mLT	-	-
15- TCI	35 μg F1-V	25 μg mLT	-	-
16- SC	10 μg F1-V	10 μl Alhy	-	-

The aerosols were generated using a Collison nebulizer (BGI Inc., Waltham, MA) supplied with 18 PSIG of HEPA-filtered air. This nebulizer generated particles with a mass mean aerodynamic diameter (MMAD) of 0.9 μm, and a geometric standard deviation (σ_g) of 1.4 (APS 3320, TSI Instruments, St. Paul, MN). The mice were challenged using a dynamic 30 liter humidity-controlled plexiglas whole-body exposure chamber. Total flow through the chamber was 19.5 l/minute and was maintained at atmospheric pressure throughout the exposure. The test atmosphere was continuously sampled using a 6 liter per minute all glass impinger (AGI; Ace Glass, Vineland, NJ). HBI media with 0.001% v/w Antifoam A (Sigma Inc., St Louis, MO) was used as impingement collection media. Nebulizer and AGI samples were plated after the exposure to establish the aerosol concentration within the exposure chamber. Using the exposure concentration, an inhaled dose was estimated by multiplying the empirically-determined aerosol exposure concentration (CFU/l air) in the chamber by the amount of air that was estimated to have been breathed by the mouse during the exposure. The cumulative air breathed by each mouse during the exposures was calculated by estimating the respiratory minute volume based on Guyton's formula and has been previously described (18).

The survival data of this challenge is depicted in Table 5 and Figure 4. In general, animals fell into three immunization groups based upon survival. Challenge Group 1 – Non-protected (0% - 20%), consisting of naïve animals (Group 1), animals receiving adjuvant only by any route (groups 3, 6), animals receiving a single priming dose IN or TC antigen + adjuvant with no boost (groups 14, 15), and animals primed IN and boosted IN with antigen but no adjuvant (group 2). Challenge Group II – Intermediate protection (30% - 70%), animals primed IN, SC, or TC and boosted SC with antigen only, no adjuvant (Groups 5, 8, 12). Challenge Group III – Protected (80% - 100%), animals primed IN and boosted IN with antigen and adjuvant, animals in any combination of prime and boost that included SC immunization with antigen and adjuvant Groups 4, 7, 9, 10, 11, 13, 16).

Table 5 – Aerosol Challenge Survival Groups

Challenge Group I (%)	Challenge Group II (%)	Challenge Group III (%)
Naïve (0)	IN x SC Antigen only (30)	IN x IN Antigen + Adjuvant (90)
IN x IN Antigen only (0)	SC x SC Antigen only (70)	IN x SC Antigen + Adjuvant (100)
IN x IN Adjuvant only (0)	TC x SC Antigen only (40)	SC x SC Antigen + Adjuvant (90)
IN x SC Adjuvant only (0)		SC x TC Antigen only (80)
IN Antigen + Adjuvant (10)		SC x TC Antigen + Adjuvant (90)
TC Antigen + Adjuvant (10)		TC x SC Antigen + Adjuvant (100)
		SC Antigen + Adjuvant (80)

The most interesting findings from these studies are that that 1) a single dose of 10 µg F1-V + alum administered SC was sufficient to protect 90% of the mice from lethal aerosol challenge even though antibody levels in those mice were significantly lower than the other immunized groups, and 2) animals primed and boosted IN with 5 µg F1-V in combination with LT(R192G) as adjuvant were solidly protected (100%). The fact that a single SC immunization with F1-V administered with alum provided significant protection became a compounding variable for analysis of any heterologous prime - boost regimen that included an SC arm. Given the fact that some combinations of prime – boost produced higher and more sustained levels of anti-F1-V IgG and IgG1, it is possible that differences in protection would become apparent in a duration study. Additional studies are currently underway to identify the threshold level of systemic antibody necessary to protect mice against aerosol challenge. It is known that SC immunization does not solidly protect non-human primates against aerosol challenge; therefore further investigation of these alternate immunization strategies is warranted. The observation that a non-parenteral route of immunization (IN x IN prime – boost) provides solid protection is also important and opens the possibility of non-parenteral immunization against plague and other aerosolized bacterial pathogens and biotoxins.

Status of Specific Aim 2. Evaluate the Immune Response to F1-V in Non-Human Primates. The original Scope of Work and timetable projected completion of Specific Aim 1 by the end of the first calendar year and completion of Specific Aim 2 by the end of the second calendar year. A number of obstacles have prevented the timely initiation of Specific Aim 2. The first was a source of antigen, F1-V. There have been increased demands on limited supplies and we were not always able to obtain sufficient quantities of this antigen from USAMRIID in a timely manner. One provision of the contract was that USAMRIID had the option of requiring a mouse challenge study before proceeding to the NHP studies. At USAMRIID's request, animals to be challenged were immunized at Tulane University and shipped to USAMRIID for challenge. There was some delay while the composition of the challenge groups was agreed upon and an additional delay waiting for a window of opportunity to open at USAMRIID. These issues were discussed with LTC Adamovicz in May 2004 and he indicated that a no-cost extension would be appropriate. The funds to complete Specific Aim 2 have been set aside and a no-cost extension of this contract will be requested.

KEY RESEARCH ACCOMPLISHMENTS

- Intranasal and subcutaneous immunization are essentially equivalent for induction of serum and BAL anti-F1-V IgG1 responses when a single booster dose is administered by the same (homologous) route.

- Heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1-V IgG1 responses.

- In no case was heterologous boosting inferior to homologous boosting and in three specific cases heterologous boosting was more effective than homologous boosting.

- IN and SC priming were more effective than TCI priming for induction of serum anti-F1-V IgG1 when the boost was administered by any route and not different from one another through six-months post-primary immunization.

- With respect to BAL responses, either IN or SC prime followed by any boosting route induced significantly higher BAL anti-F1-V IgG1 than TCI priming, at least through six-months post-primary immunization, clearly demonstrating that either IN or SC priming may be effective when a bronchioalveolar response is desired.

- As single SC immunization with F1-V alone, with or without alum as an adjuvant, was sufficient to protect mice against aerosol challenge with 70 LD50 of *Y. pestis* CO92.

- IN prime and boost with LT(R192G) as an adjuvant provided solid (100%) protection against aerosol challenge with 70 LD50 of *Y. pestis* CO92.

REPORTABLE OUTCOMES

Manuscripts submitted for publication Glynn, A, Freytag, L.C., and Clements, J.D. 2004. Effect of homologous and heterologous prime - boost on the immune response to recombinant plague antigens. Vaccine (Submitted for Publication).

Abstracts: Glynn, A, Freytag, L.C., and Clements, J.D. 2004. Enhancing the immune response to recombinant plague antigens. Presented at the 104th Annual Meeting of the American Society for Microbiology, New Orleans, LA

CONCLUSIONS

In the current study, we examined different prime - boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the effect of changing the route of prime and boost on the ability of recombinant F1-V to promote the development of long-lasting, high titer antibodies. We also examined the effect of different prime - boost regimes on the compartmentalization of the ensuing immune response.

The most significant findings of the immunization study are that 1) IN and SC immunizations are both effective and essentially equivalent for induction of serum and BAL anti-F1-V IgG1 responses when a single booster dose is administered by the same (homologous) route, 2) heterologous boosting can be as or more effective than homologous boosting for induction of either serum or BAL anti-F1-V IgG1 responses, and 3) anti-F1 and anti-V total IgG responses were highest in animals primed IN and boosted by any route when compared to animals primed TC or SC. As with previously published studies, there were still detectable levels of circulating anti-F1-V antibodies even one year post-primary immunization.

The observation that heterologous boosting may, in some cases, produce higher and more sustained antibody responses than homologous boosting is consistent with other reports that have examined this question (1, 14-17, 20). Most recently, Lauterslager et al. (14) demonstrated that oral (PO) boosting with ovalbumin was more effective in animals primed IN, SC, or intraperitoneally than PO (homologous) boosting with the same antigen. Similarly, Nicholas et al. (16) demonstrated that SC immunization was effective for priming animals subsequently boosted

SC or IN (but not PO) with chimeric virus particles expressing a 17-mer peptide sequence from canine parvovirus (CPMV). In those studies, animals primed IN and boosted SC developed significantly higher serum anti-CPMV IgG2a responses than did animals primed IN and boosted IN. In the study of Baca-Estrada et al. (1) mice immunized SC with formalin killed whole cells and then boosted IN with formalin killed whole cells, either alone or formulated in liposomes, developed higher serum and BAL anti-*Y. pestis* antibody and higher systemic cell-mediated immune responses than did animals boosted SC. Our findings are in agreement with these studies, all of which demonstrate that heterologous boosting can be as or more effective that homologous boosting for induction of serum antibodies. Since serum IgG1 has been shown to be protective against aerosolized *Y. pestis*, mucosal IgA was not examined in the current study. However, Baca-Estrada et al. (1), Lauterslager et al. (14), and Nicholas et al. (16) each demonstrated that heterologous boosting could also induce significant mucosal IgA responses.

The fact that IN and TC boosting of SC-primed animals generated higher levels of anti-F1-V antibodies than homologous SC boosting is interesting and could be explained by the distribution of T effector-memory cells to the peripheral tissues following SC priming where they would be available to interact with cognate antigen applied mucosally or transcutaneously in the context of an appropriate adjuvant (e.g., LT(R192G)) (3). However, SC boosting of IN and TC primed animals was also more effective than homologous IN or TC boosting for induction of serum ati-F1-V antibodies, suggesting that a more global immunological phenomenon may be functioning here. Moreover, the adjuvant employed for IN and TC immunizations may also play a role. A number of studies have shown that the ADP-ribosylating enterotoxins can induce phenotypic and functional maturation of dendritic cells as well as interacting directly with T-helper cells, B-cells, and epithelial cells. Both the Lauterslager et al. (14) and Nicholas et al. (16) utilized cholera toxin as a mucosal adjuvant. Clearly, the role of the adjuvant in controlling these outcomes requires further investigation.

The amount of antigen delivered by each route may also contribute to the outcome. Thus, the observation that both IN and SC regimens induced significantly higher levels of serum and BAL anti-F1-V IgG1 than did TC immunization with the same antigen (Fig. 2) may be influenced by the amount of antigen delivered. It is possible that the responses to TC prime and boost would be higher if larger amounts of antigen were applied or other methods were used to make uptake of transcutaneously administered antigens more efficient.

In and of itself, the observation that immunization by one route can prime for a secondary response by another route is important. In practical terms alone, especially in an imminent or post-release bioterrorism event, the ability to administer a parenteral priming dose and, at the same time, distribute a follow-on patch, pill, or nasal applicator that could be self-administered would greatly improve national preparedness.

The aerosol challenge studies also revealed two important findings: 1) a single dose of F1-V + alum administered SC was sufficient to protect 90% of the mice from lethal aerosol challenge even though antibody levels in those mice were significantly lower than the other immunized groups, and 2) animals primed and boosted IN with F1-V in combination with LT(R192G) as adjuvant were solidly protected (100%). While there was some indication in the literature that a single high dose of F1-V delivered IM or SC could provide protection against challenge, there was

no clear indication that a single, relatively small dose (10 μg) would provide 90% protection. As mentioned above this turned out to be a compounding factor in interpreting the results of the aerosol challenge study.

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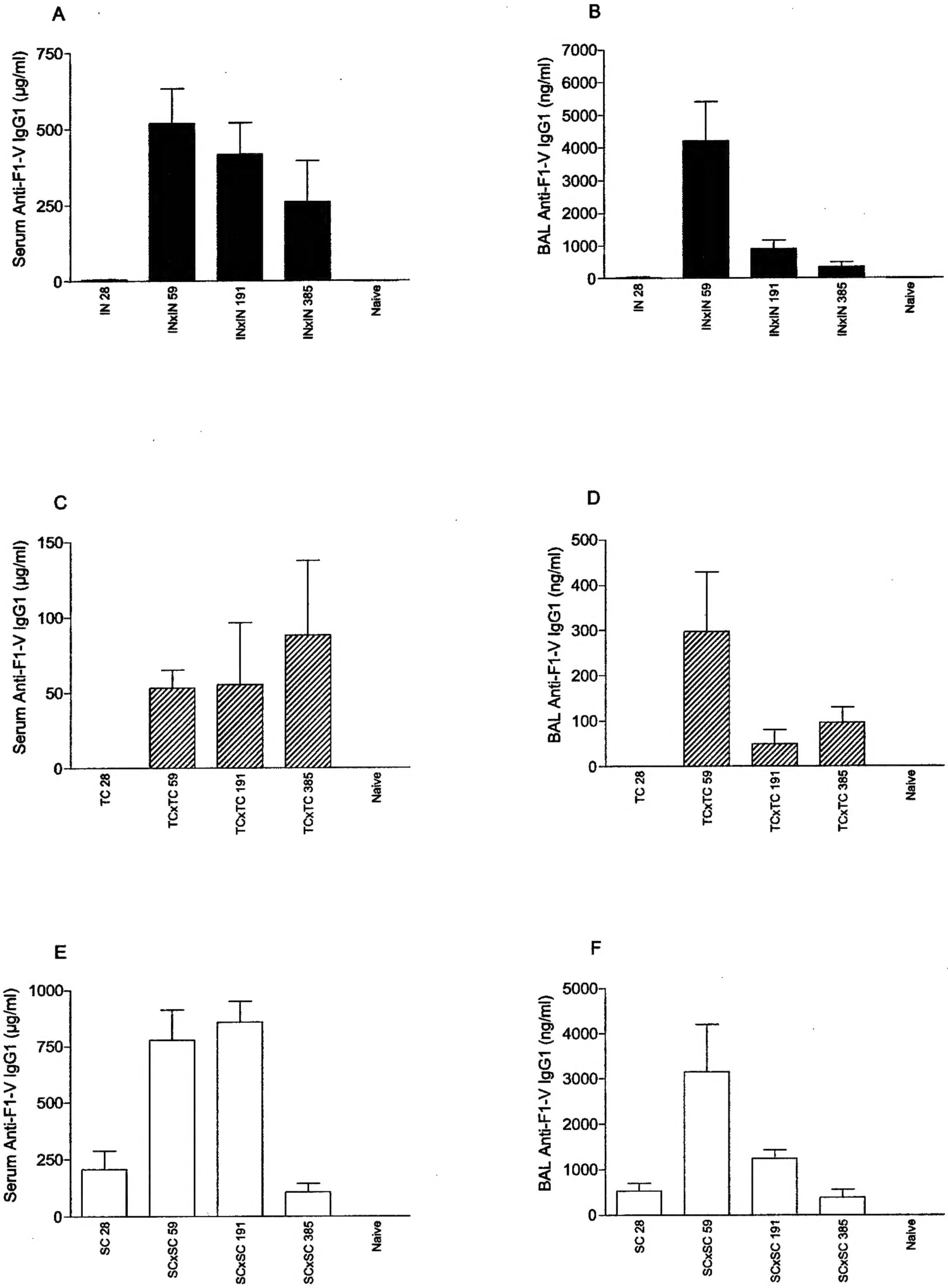

Figure 1. Swiss/Webster mice were primed IN (panels A and B), TC (panels C and D), or SC (panels E and F) on day 0 and then boosted by the same (homologous) route on day 28. Five animals from each group were sacrificed on days 28 (no boost), 59, 191, and 385 following the primary immunization. Blood was collected by cardiac puncture (serum: panels A, C, and E) and lung washes were collected by bronchioalveolar lavage (BAL: panels B, D, and F). Concentrations of IgG1 were determined by ELISA by non-linear regression against an IgG1 standard curve.

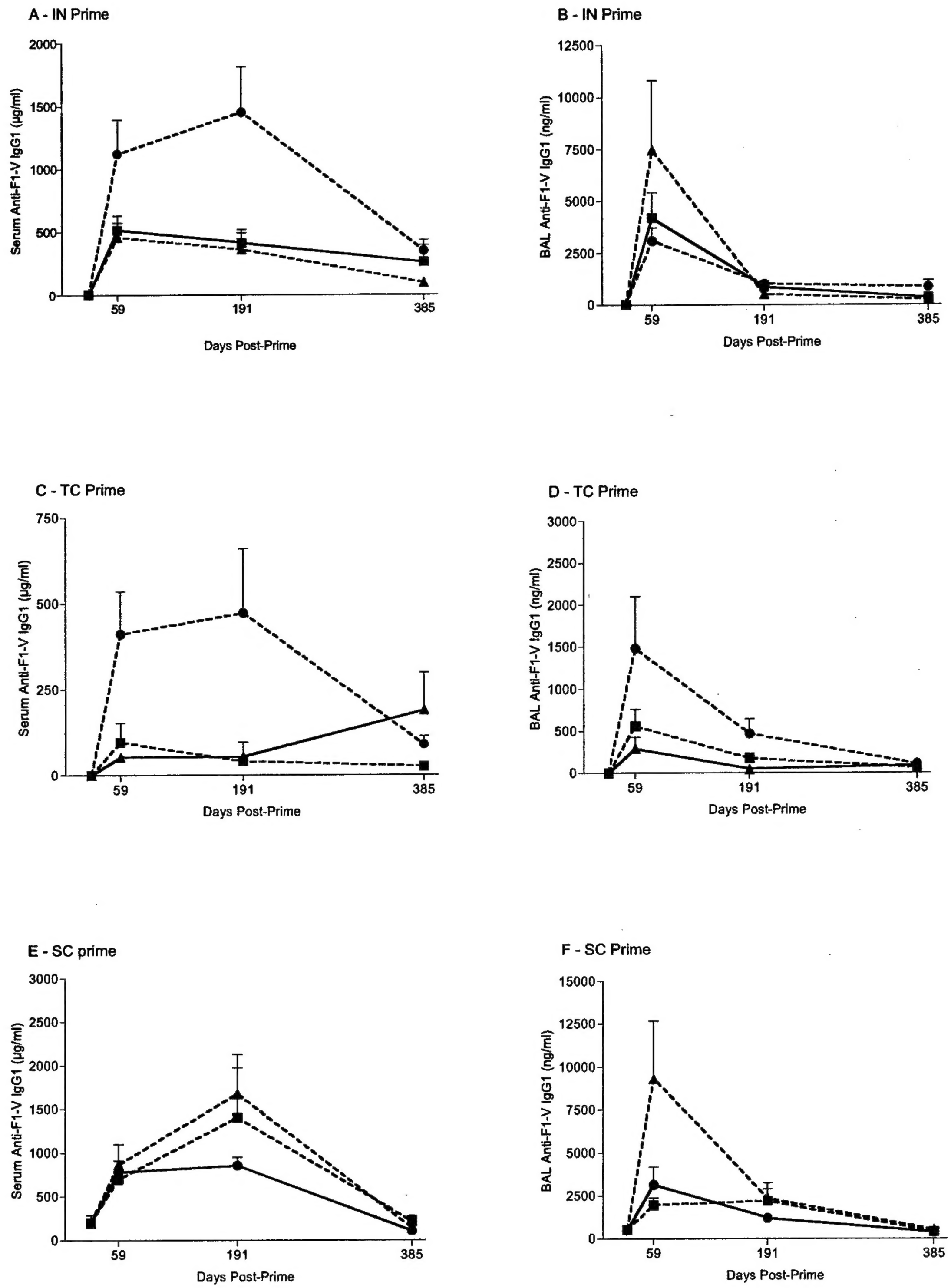

Figure 2. Swiss/Webster mice were primed IN (panels A and B), TC (panels C and D), or SC (panels E and F) on day 0 and then boosted IN (closed rectangle), TC (closed triangle), or SC (closed circle) on day 28. Five animals from each group were sacrificed on days 28 (no boost), 59, 191, and 385 following the primary immunization. Blood was collected by cardiac puncture (serum: A, C, and E) and lung washes were collected by bronchioalveolar lavage (BAL: B, D, and F). Concentrations of IgG1 were determined by ELISA by non-linear regression against an IgG1 standard curve. Solid lines represent homologous boosting. Dotted lines represent heterologous boosting.

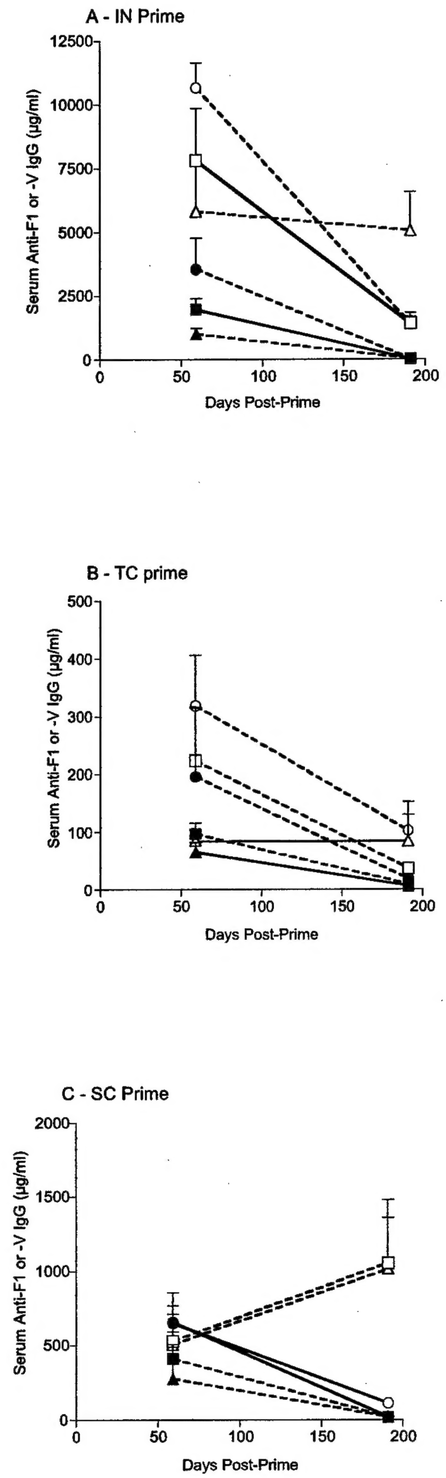

Figure 3. Swiss/Webster mice were primed IN (panel A), TC (panel B), or SC (panel C) on day 0 and then boosted IN (rectangle), TC (triangle), or SC (circle) on day 28. Serum was analyzed from five animals in each group sacrificed on days 59 and 191 following the primary immunization. Concentrations of IgG were determined by ELISA by non-linear regression against an IgG standard curve. Open symbols are serum anti-V IgG. Closed symbols are serum anti-F1 IgG. Solid lines represent homologous boosting. Dotted lines represent heterologous boosting..

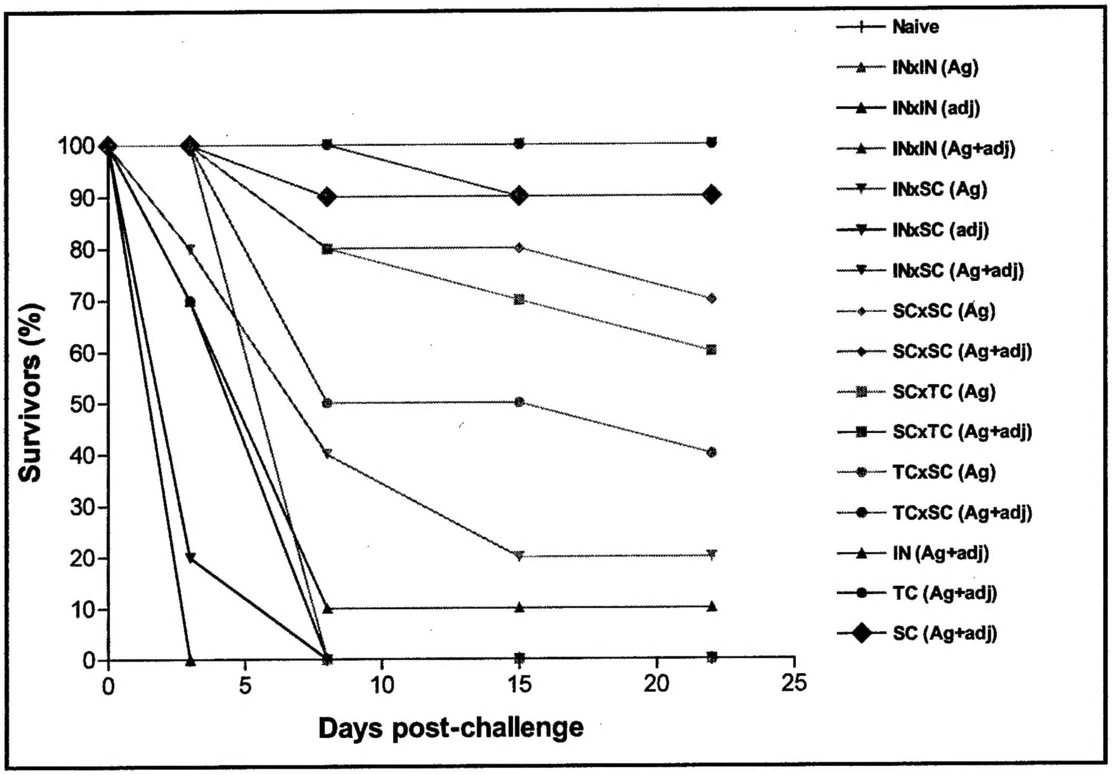

Figure 4. Sixteen groups of ten 6-8 week old female Swiss Webster mice were immunized once (day 0) or twice (day 0 and day 28) with recombinant F1-V with and without adjuvant (Table 1). Mice were challenged on day 87 post-primary immunization by aerosol with 70 LD50 of *Y. pestis* (Colorado 92) and monitored for survival.